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14. ABSTRACT

Lasers generating predominantly thermal energy are used in medicine and research for a variety of purposes including surgical excision, pan retinal photocoagulation for treating diabetic retinopathy, corneal shape remodeling, treatment of photoaged skin, and hair removal. Not surprisingly, there has been an increase in the number of laser injuries, especially eye injuries, due to laser misuse or accidents over the last four decades. When sufficient energy is provided, most visible and near infrared wavelength laser systems will damage the retinal pigment epithelium (RPE). This damage is generally due to thermal injury. Of particular concern is thermal laser injury to the macular region of the retina, which may result in a blinding trauma that produces an immediate psychological and physical debilitation.

To provide rational treatments for laser-induced injury, a better understanding of the nature of this injury is required. To this end, we established methods for studying laser-induced injury with in vitro models utilizing human cells. Cultured human cells provide

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In vitro Models of Laser Induced Injury: Pathophysiology and Cytoprotection

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In vitro Models of Laser Induced Injury: Pathophysiology and Cytoprotection

Introduction

Lasers generating predominantly thermal energy are used in medicine and research for a variety of purposes including surgical excision (Barrett et al., 1996; D.H. Sliney & Wolbarsht, 1989) pan-retinal photocoagulation for treating diabetic retinopathy, corneal shape remodeling, treatment of photoaged shin (Anderson, 2000; Chiarelli & Muglia, 2001), and hair removal (Ash, Lord, Newman, & McDaniel, 1999; Bargman, 1999; Baugh, Trafeli, Barnette, & Ross, 2001; Bencini, Luci, Galimberti, & Ferranti, 1999). Not surprisingly, there has been an increase in the number of laser injuries, especially eye injuries, due to laser misuse or accidents over the last four decades (Rockwell, 1994). When sufficient energy is provided, most visable and near infrared wavelength laser systems will damage the retinal pigment epithelium (RPE). This damage is generally due to thermal injury (Marshall & Mellerio, 1967b; D. Sliney & Wolbarsht, 1980; D. H. Sliney & Wolborsht, 1980). Of particular concern is thermal laser injkury to the macular region of the retina, which may result in a blinding trauma that produces an immediate psychological and physical debilitation (Mellerio, Capon, Docchio, Sliney, & Krafft, 1988).

Laser-induced ocular damage is a particular concern for the military, as many such casualties would require large numbers of medical personnel to care for them. Accidental ocular exposures to military laser devices have been documented. There currently exist more than 200 military laser systems already in use or in various stages of development in at least 50 different

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countries. Some of these systems may be under development as anti-personnel or anti-eye systems and thus present a hazard to the soldier. Therefore in countering such threats, it has become important to develop medical management strategies to enhance military medical readiness for military operational medicine and combat casualty care.

Laser light energy interacts with living cells in a variety of ways. Photochemical damage caused by wavelength-specific interaction of light with biological molecules can be produced by ultraviolet lasers such as the argon fluoride excimer (193nm), the krypton fluoride excimer (KrF, 248 nm), the xenon fluoride excimer (XF, 308nm), and to a lesser extent by continuous wave (CW) blue light lasers such as HeCd (441 nm) and argon (488 nm). Photoionization or photomechanical effects resulting from Q-switched and mode-locked systems pulsing on the 20 nanoseconds to 50 femtoseconds time domains are produced by laser systems such as the Nd:YAG (1064 nm), the frequency doubled Nd:YAG (532 nm), the titanium-sapphire (800-900 nm), and Nd:glass (1060 nm). Thermal effects are typically produced by CW laser systems such as the argon (514.5 nm), the frequency doubled Nd:YAG (532 nm), dye lasers (577- 610 nm), krypton lasers (530, 568, and 647 nm), and infrared lasers such as the chemical oxygen iodide (COIL, 1.3um), hydrogen-fluoride (HF, 3.0 um), and carbon dioxide (10.6 um). For example, at high CO_2 laser irradiances the temperature of water can rise to boiling with greater than 99% of the absorbed radiation located in the first 50 microns of water within the tissue (Mainster, 1986). The thermal effects of lasers are readily recognized and are in many cases the principal mechanism for laser-related injury. These same thermal effects are often exploited to beneficial effect in medical applications.

In the effort to better understand laser-tissue interactions, a variety of animal models have been examined (Marshall & Mellerio, 1970; Schirmer, 1992; D. H. Sliney, 1985; A. J. Welch,

1984). Much has been learned about how laser light can damage biologic tissue, especially the retina. However, most of what we know from animal models is based on morphological data (Mainster, Crossman, Erickson, & Heacock, 1990; Mainster, White, & Allen, 1970; Mainster, White, Tips, & Wilson, 1970; Marshall & Mellerio, 1968; Marshall & Mellerio, 1967a, 1967b, 1970). Because the injuries are small and there are poor signal-to-noise ratios of any released biochemical/molecular signals, biochemical and molecular data have not been obtained. Extensive biochemical studies in animal models are also expensive, time consuming, and allow evaluation of only a few time points. For all of these reasons, it has been difficult to formulate therapeutic measures based on a thorough understanding of the underlying injury.

To provide rational treatments for laser-induced injury, a better understanding of the nature of this injury is required. To this end, we established methods for studying laser-induced injury with in vitro models utilizing human cells. Cultured human cells provide an opportunity to study a process at the cellular level and derive fundamental information about basic human cellular responses to injury or drugs. One important limitation is that in vitro models lack whole organism cellular and physiological interactions and thus represent only a subset of all possible cellular responses. To partially address this limitation, we have investigated thermal injury in a variety of cell types comparing damage caused by heated media and lasers. We have also evaluated therapeutic means to ameliorate thermal trauma. Our in vitro approaches serve as screening methods to identify the most promising therapeutics for transitioning into animal models.

Background

Laser-induced injuries typically involve focal damage to external tissues such as the skin and cornea. Visible and near-infrared laser wavelengths can thermally damage internal tissues

such as the retina. Lasers typically deliver photon energy with a Gaussian energy distribution, usually as small spots (25-500 um diameter) with greater intensity in the center than at the edges. Thus, the resulting injury is not uniform. The study of laser-tissue interactions in vivo and in vitro has been limited by the non-uniformity of laser-induced injury. When sufficient energy is used, most of the cells across the diameter of the beam are killed. Only a small percentage of cells are responders that are available for study. Thus, we have been limited in our ability to study the cellular effects of laser thermal injury because it is difficult to obtain a sufficient amount of cellular material for biochemical analysis. We recently described a raster scanning technique that uses a computer to san a laser beam across a cell layer that provides for a uniform distribution of energy (Walker, Schuschereba, Edsall, Stuck, & Bowman, 2007) but most of the results to be described were obtained by surrogate techniques to mimic a laser irradiation.

Figure 1A illustrates the problems of the nonuniform distribution of energy across the beam profile. Following laser irradiation with a carbon dioxide laser (10.6 um), the cell layer was treated with MTT for 1 hour and then photographed. A purple formazan is deposited in viable cells while it is absent in dead cells and reduced in injured cells. A hormetic effect, i.e. a low-dose effect that appears to be opposite to that caused by high doses, can be seen in the outer most ring of the area of irradiation (Calabrese et al., 2007; Scannapieco, Sorensen, Loeschcke, & Norry, 2007). The cells in the center of the lesion are heat-fixed and have undergone accidental cell death. A small number in the periphery are in the process of undergoing apoptotic cell death, and cells responding to hormesis are on the periphery and exhibit increased MTT reduction to formazan. Figure 1B illustrates this effect at higher magnification. The punctate pattern of formazan distribution represents the production of formazan within mitochondria. Figure 1C presents immunocytochemical staining for heat shock protein 70 (hsp70 and further

demonstrates that a single exposure of cells to a laser via an aperture only produces a small area or responding cells and that most cells are either killed or are not effected by the laser.

⇒FIGURE 1

That the injury depicted in Figure 1C is predominantly thermal was demonstrated in early work with the carbon dioxide laser (10.6 μm). Millisecond exposures to a carbon dioxide (CO_2) laser induced the production of hsps in sublethally injured human fibroblast (WI-38) cell cultures (Ferrando, Schuschereba, Quong, & Bowman, 1995; Schuschereba et al., 1994) (Figure 3). Heat shock or stress proteins are induced to high levels within minutes to hours of exposure to temperature elevation of a few degrees above normal. These proteins are highly conserved throughout nature and their wide range of functions is just beginning to be understood. Their induction by sublethal doses of laser energy suggests that thermal injury is an important component of this injury. Infrared thermography during irradiation also indicated that laser irradiation elevated the temperature to about 67 ± 2°C for 800 milliseconds (Ferrando et al., 1995). This result corresponded well to Moritz and Henrique's estimation that the basal layer of the human epidermis was killed by exposure to 65°C for one second (Moritz & Henriques, 1947).

By far, the most extensive medical experience with thermal injuries has been with burns. In the United States, more than 2 million people suffer burn injuries each year, resulting in 6,500 burn and fire-related deaths annually (Pruitt, 1991). However, few studies have examined the cellular response to thermal injury either in terms of the cellular molecules that are most affected by burn injury or how to ameliorate the sequelae of burn injury. This lack of basic scientific information may be due to the fact that burns are considered to be avoidable accidents, rather than diseases which pose a threat to the public at large. However, from the military perspective,

thermal injuries may not be avoidable, particularly if they can be inflicted intentionally by adversaries equipped with laser weapon systems.

Normal Human Epidermal Keratinocytes (NHEK)

Some information about the hyperthermic death of cells exists in the context of the elimination of cancerous tissue by exposure to temperatures in the range of 40-47°C (Dewey, 1979, 1984; Rice, Gray, Dean, & Dewey, 1984; Rice, Gray, & Dewey, 1984; Sapareto & Dewey, 1984). However, most of these in vitro cancer studies have used established Chinese hamster ovary cells and have employed clonigenic assays to determine cell survivability. Such studies are difficult to interpret in terms of the kinetics of cell death and survival, and there have been no biochemical studies to determine the mechanism of death or survival. It seems reasonable that different cells and tissues may be differently sensitive to heat. Little is known about how normal human cells might respond when exposed to short duration high temperatures. To address this issue, we devised a method of exposing cultured human cells to thermal injury by growing them on coverslips or in small culture dishes. Cells were then dipped into a relatively large volume of heated saline for up to 30 seconds and viability assays were utilized.

Normal human epidermal keratinocytes (NHEK) provide a good model for cells that cover the human body, including the cornea. In previous studies, these cells were monitored for survival, apoptosis, and hsp production after 4-second exposures over a temperature range of 52-62 ° C (Figure 2). The in vitro temperature gradient followed expected temperature gradients across a Gaussian energy profile of a laser beam for a carbon dioxide laser.

⇒FIGURE 2

Studies of the heat shock or stress response have focused generally on the effect of elevating temperatures a few degrees above physiological for minutes to hours at a time. Few studies have addressed the consequences of brief exposure to relatively high temperatures. Figure 2 illustrates the survival curves for NHEK subjected to different high temperatures for 1-second exposure periods. These were analyzed for survival as a function of time after treatment (Bowman et al., 1997). Generally, the higher the temperature of exposure, the more rapid is cell death. An upper limit of 58°C for one second is lethal for all keratinocytes. These cells can tolerate exposures to 55°C for 6 seconds, but no cells can survive above 65°C. At different times after heating, cells were assayed for viability with a modification of the MTT method described by Mosman (Mosman, 1983). Survivability was presented as the percentage of cells surviving relative to the untreated control. The results showed that for temperatures above 62°C, few cells were alive at six days.

Figure 3 depicts an Arrhenius plot for heat inactivation as measured by cell viability. The Arrhenius plot defines changes in reaction rates (cell inactivation as a function of temperature) and thus allows us to determine the activation energy for cell responses. The changes in slope at 51° C and 59° C indicate that major transitions occurred in the molecular species inactivated by heat for this cell type and heating regimen (Klavuhn & Green, 2002; Kruuv et al., 1983; Lepock & Kruuv, 1980; Mackey, Morgan, & Dewey, 1988; Raaphorst, Feeley, Chu, & Dewey, 1993). Such transition points in slope could also be interpreted as changes in the mechanism of inactivation.

⇒FIGURE 3

Heat Shock Protein Synthesis

The heat shock response was first described by Ritossa in 1962 (Ritossa, 1962) based on observations of gene activity in Drosophila polytene chromosomes. This response has been observed subsequently in all species studied. If pre-induced, the heat shock response is very cytoprotective against lethal cell injury. Heat shock protein induction is usually studied by exposing cells to temperatures between 41°C and 45°C for 15 minutes to 1 hour. The protein product of the heat shock genes are a subset of a larger group of proteins known as molecular chaperones, which serve to protect proteins from inappropriate aggregations (Papp, Nardai, Soti, & Csermely, 2003; Parcellier, Gurbuxani, Schmitt, Solary, & Garrido, 2003). This function is important under normal conditions, but assumes additional importance when denaturation of protein is accelerated during periods of stress. Heat is a prominent protein denaturant, as are ischemia, heavy metals, ethanol, and certain drugs (Lindquist, 1986; Lindquist & Craig, 1988).

We demonstrated in keratinocytes that very brief exposure (1-5 seconds) to relatively high temperature (56-60 °C) could induce heat shock protein synthesis (Bowman et al., 1997). After SDS polyacrylamide gel electrophoretic separation of proteins from NHEK, Western blots immunostained for hsp70 were obtained and examined 8 hours after exposure (Figure 4). Short exposures of NHEK to high temperatures induced hsp70 synthesis in a dose-dependent manner up to a maximum temperature at which synthesis abruptly ceased. This temperature was inversely related to the duration of exposure and closely followed the survival data (Figure 4). Staining for hsp90 and hsp27 showed that their levels were not elevated above control (data not shown).

⇒FIGURE 4

Synthesis and Release of IL-8

Garner et al (Garner et al., 1994) first showed that large amounts of interleukin-8 (IL-8) were released during a 24-hour period by organ cultured second degree burn tissue relative to uninvolved control skin. We therefore examined IL-8 synthesis by NHEK after thermal injury to see if this response could be duplicated with a pure population of skin cells. The release of IL-8 into the medium was examined by enzyme-linked immunoassay. During the 24 hours following a 4-second exposure to temperatures between 52° C and 56°C, we observed a dose-dependent release of IL-8 into the medium (Figure 5). The greatest amount of IL-8 was observed after exposure to 56°C. In sharp contrast, no release was observed after exposure to temperature of 57°C or higher. These data indicate that synthesis and release of IL-8 into the surrounding injury site occurs after the thermal injury, and that this is probably mediated by pro-inflammatory events.

⇒FIGURE 5

Figure 6 summarizes that relationship between temperature, survivability and hsp70 production and apoptosis in HEHK for a 4 second exposure to heat, The cytoprotective effect of hsp70 is first induced but as the temperature continues to rise, it is insufficient to counteract the effects that are leading to apoptosis cell death.

⇒FIGURE 6

Apoptosis and Accidental Cell Death after Thermal Injury

Apoptosis, sometimes called programmed cell death, has received considerable attention because there is evidence to indicate that injured cells can program or regulate this response, and thus may be reprogrammed. The respective roles of apoptosis and necrosis or accidental cell death (ACD) (Majno & Joris, 1995) (also see oncosis, chapter 3) after thermal injury were evaluated in NHEK (Matylevitch et al., 1998). Following the recommendation of Majno and

Joris (Majno & Joris, 1995) and Trump (Trump, Berezesky, Chang, & Phelps, 1997), we reserve the term apoptosis for cell death in cells with intact plasma membrane, mitochondria, and protein synthetic apparatus.

The importance of apoptosis in regulating cellular homeostasis through control of cell death is undisputed. However, it is not a simple task to discriminate an apoptotic cell from one that is dying from ACD (Majno & Joris, 1995). Morphological features of apoptosis such as nuclear fragmentation are readily recognizable at the light microscopic level, but are present only for a short time and only during the final stages of the process. Progression toward the final execution stage does not occur synchronously in all cells. Thus, it is not sufficient to apply a particular biochemical assay. While both apoptosis and ACD lead to degradation of nuclear DNA, only apoptosis is gene-directed and thus possibly preventable. To the extent that apoptosis occurs after thermal injury, it may present a unique opportunity for cell rescue and improvement of cell healing after burn injury. No treatment can save cells that are undergoing ACD. Thus, the potential benefit of preventing still-viable cells from undergoing apoptosis will require the development of methods to discriminate between apoptosis and ACD. The terminal deoxynucleotidyl-mediated dUTP nick end-labeling (TUNEL) method developed by Gavrieli (Gavrieli, Sherman, & Ben-Sasson, 1992) is often used to evaluate apoptosis in vivo and in vitro. TUNEL can provide an early indication that DNA fragmentation is occurring. However, the method is not specific. Cells undergoing ACD can also be labeled by the TUNEL method (Cervos-Navarro & Schubert, 1996; Charriaut-Marlangue & Ben-Ari, 1995; de Torres, Munell, Ferrer, Reventos, & Macaya, 1997; Grasl-Kraupp et al., 1995; Nakamura, Sakai, & Hotchi, 1995; van Lookeren Campagne & Gill, 1996). Therefore, information about cell viability is

necessary to accurately determine whether cell death is due to apoptosis or to ACD (Dypbukt et al., 1994).

NHEK were evaluated after heating to temperatures of up to 72°C for 1 second (Matylevitch et al., 1998). After determining whether a cell was viable by the LIVE/DEAD™ fluorescent viability assay, the TUNEL assay was performed on the same cells. Apoptosis was defined as nuclear degradation occurring within viable cells; TUNEL-positive cells were considered to be in apoptosis if they were determined viable by the LIVE/DEAD™ assay. By coupling the LIVE/DEAD™ fluorescent viability assay with the TUNEL method and ultrastructural morphology, apoptosis and ACD could be distinguished (Figure 7). After exposure to temperatures of 58-59°C, cells died predominantly by apoptosis; viable cells became TUNEL positive indicating degradation of DNA (Figure 7A- D). After exposure to temperatures of 60-66°C, we observed TUNEL positive viable and nonviable cells, indicating that apoptosis and ACD were occurring simultaneously (Figure 7 E-H). Depending on temperature and time of exposure, NHEK died either by apoptosis or ACD. After exposure to temperatures above 72°C, cells died almost immediately, presumably from heat fixation (Figure 7 I-J).

⇒FIGURE 7

Mitochondria appear to play a prominent role in apoptotic cell death. The relationship between thermal injury and mitochondrial permeability transition is reported to be a standard feature of apoptosis (Hirsch, Marzo, & Kroemer, 1997; Kroemer, Zamzami, & Susin, 1997; Lotem, Peled-Kamar, Groner, & Sachs, 1996; Macho et al., 1996; Magal, Jackman, Pei, Schlegel, & Sherman, 1998; Marchetti et al., 1996; Petit et al., 1997). In our study, fluorescent mitochondrial probe MitoTracker™ Orange, developed by Kroemer (Macho et al., 1996),

indicated that cells undergoing apoptosis became TUNEL positive prior to the loss of mitochondrial function (Figure 8).

⇒FIGURE 8

Nuclear breakup or nucleosomal fragmentation of DNA occurred after exposure to 58-59°C (Figure 9). By transmission electron microscopy, the characteristic morphologic findings of cells undergoing apoptosis were observed to include cellular shrinkage (Figure 9A), nuclear condensation (Figure 9B, 9C), cytoplasmic budding (Figure 9D), and relatively intact mitochondria (Figure 10).

⇒FIGURE 9

⇒FIGURE 10

The loss of mitochondrial transmembrane potential preceded overt nuclear signs of apoptosis. This may be used instead of the LIVE/DEAD assay in conjunction with TUNEL to discriminate between apoptotic and ACD in a variety of cell types. Permeability transition precedes nuclear fragmentation and involves opening of the mitochondrial megachannels, allowing free distribution of solutes < 1500 Da on both sides of the inner mitochondrial membrane (Hirsch et al., 1997; Kroemer et al., 1997; Lotem & Sachs, 1996; Macho et al., 1996; Magal et al., 1998; Marchetti et al., 1996; Petit et al., 1997). This membrane alteration results in loss of the proton gradient and uncoupling of oxidative phosphorylation. Since the dye can be covalently bound to cells with aldehydes, it could be assessed after fixation and used in conjunction with the TUNEL assay. A viability assay would then not be required, nor would the same area of a specimen need to be evaluated a second time.

Summary of Apoptosis and Accidental Cell Death

Several studies have shown that skin keratinocytes undergo apoptosis during normal development and also as part of their program of terminal differentiation (Dypbukt et al., 1994; Polakowska, Piacentini, Bartlett, Goldsmith, & Haake, 1994). Work with NHEK describes the induction of apoptosis in normal human keratinocytes after a short pulse of relatively high temperature. These results are consistent with other findings that the mode of cell death is a function of the intensity of the insult (Bonfoco, Krainc, Ankarcrona, Nicotera, & Lipton, 1995; Dypbukt et al., 1994). Very high temperature exposure results in immediate cell death by a form of heat fixation.

An effective method for discriminating between apoptosis and ACD couples a viability assay with a DNA damage test. This method can be used to demonstrate that the mode of cell death is gene directed and is thus apoptosis. If the cell is alive and TUNEL positive, it is probably undergoing apoptosis. If a dead cell is TUNEL positive and exhibits neither nuclear nor cytoplasmic fragmentation, its death was probably due to ACD.

Most cell types exhibit morphological features of apoptosis such as decreased cell volume and marked shape changes with budding. By electron microscopy, apoptotic cells exhibit relatively intact but shrunken mitochondria, aggregation of chromatin, break up of the nucleus into apoptotic bodies, and generation of pseudopodia (budding). These characteristics were also observed here after thermal injury to NHEK following thermal exposure in the range of 57-59°C. The degradation of DNA to nucleosomal size fragments was a very late event in keratinocytes exposed to 58°C and 59°C thermal injuries. In this case, DNA degradation occurred perhaps at or about the time the cells rounded up and detached from the substrate. Phagocytosis by neighboring keratinocytes was attempted but probably not completed. In this model, condensation of chromatin and fragmentation of nuclei were late events in apoptosis.

Following exposure to temperatures above 60°C, ACD was the predominant mode of cell death; most cells were ethidium homodimer positive before they became TUNEL positive. This pattern did not change much after treatment at 62-66°C, although cells detached more rapidly at these higher temperatures and this may have reflected denaturation of cellular integrins or extracellular matrix attachment proteins. Cell loss into the medium at these higher temperature exposures was high and it was therefore difficult to quantify changes in viability and TUNEL staining in these cells.

Although apoptosis is considered to be gene directed, the nature of the expressed genes is largely unknown. The capase family of cysteine proteases play a prominent role in apoptosis. They are preformed proenzymes and they act upon each other in a proteolytic cascade once apoptosis is triggered; they do not have to be synthesized. We observed that hsp70 levels were elevated after exposure to temperatures up to 58°C for a 1-second exposure, but they were not synthesized at higher temperatures.

We also examined the ability of thermally injured NHEK to synthesize phosphoprotein p53, which appears to play a pivotal role in apoptosis (Lotem et al., 1996; Magal et al., 1998). Although the mechanism is yet unknown, p53 is best known as a tumor suppressor that accumulates after DNA damage. It has been suggested that p53 may play a role in the induction of apoptosis in ultraviolet light irradiated keratinocytes (Henseleit et al., 1997). Although thermal injury is not known to induce DNA damage directly, we observed that p53 synthesis was elevated above control in thermally injured, yet viable cells. This occurred after exposure to 58°C, but not at higher temperatures. Cells heated to > 59°C probably cannot undergo apoptosis and thus die by ACD. Our results are consistent with the role of p53 in inducing apoptosis.

The results reported here strongly suggest that apoptosis occurs in epidermis in vivo after burn injury when the basal cell layer is heated to 58-59°C, and further that ACD occurs after injury from higher temperatures. Wound healing could be accelerated by treatments to rescue thermally injured, yet viable keratinocytes. We have focused on human epidermal keratinocytes, but similar results have been obtained with normal human umbilical vein endothelial cells, HeLa cells and cultured retinal pigment epithelial cells (unpublished observations).

Cytoprotection with Heat Shock

Induction of heat shock proteins following a sublethal exposure to heat provides cytoprotection that leads to a significant increase in the survival of cells exposed to a subsequent lethal thermal injury. A number of animal studies have shown protective effects of heat pretreatment prior to a variety of injuries such as ischemia/reperfusion and hemorrhage. Of course, the animals in these studies are anesthetized as core body temperature is elevated to 42°C for 15-60 minutes, followed by a wait of at least 4 hours for development of the response. Most in vitro studies have focused on the effect of elevating temperature a few degrees above physiological for minutes to hours. Few studies have addressed the consequences of brief exposure to relatively high temperatures as might occur during thermal injury. We found that pretreatment of NHEK with short exposure to relatively high temperatures conferred thermotolerance to the cells. The pretreated group was heat shocked at 55°C for 4 seconds. Six hours later, this and a control group of cells were heated for 4 seconds to various temperatures and the MTT assay was performed after 48 hours. Pretreatment of NHEK provided significant protection from the subsequent exposure (Figure 11). For example, cells pretreated with heat exhibited 50% greater viability at 62°C than their untreated counterparts.

⇒FIGURE 11

Although the heat shock response is the best studied response to thermal injury and can perhaps be explained as an adaptive, protective response to heat and stress (Dinh, Zhao, Schuschereba, Merrill, & Bowman, 2001; Henle & Warters, 1982; Levinson, Opperman, & Jackson, 1980; C. G. Li, 1982; Maytin, Wimberly, & Anderson, 1990; Nover, 1984; Subjeck, Sciandra, & Shyy, 1985; W. J. Welch & Suhan, 1985), other adaptive responses may also be involved. Understanding how cells respond to a sublethal, survivable thermal injury may provide new means to enhance protective cellular responses and increase survival. The induction of thermotolerance, as evident by the heat shock response, has been shown to provide temporary protection against ensuing episodes of injurious thermal exposure (Bowman et al., 1997; Dinh, Stavchansky, Schuschereba, Stuck, & Bowman, 2002; Dinh et al., 2001; Morimoto & Santoro, 1998). Analysis of an Arrhenius plot for the inactivation of keratinocytes (Figure 3) from short exposure (seconds) to high temperatures (50–60°C) indicated that the responsible absorbing macromolecule may be different, leading in turn to a somewhat different injury mechanism than suggested in the most studied range (40–50°C over minutes of exposure to elevated temperature). Above a certain threshold of heating, all cells die from exposure to short-duration thermal injury, but they express large amounts of hsps and cytokines before doing so. Human keratinocytes exposed for 1 second at 58°C synthesized IL-8 as well as mRNA for tumor necrosis factor-α and IL-1α and exhibited an intense heat shock response. Depending on the intensity of the heating, the initial form of cell death was apoptotic but quickly became ACD as the intensity of heating increased (Bowman et al., 1997; Matylevitch et al., 1998).

Summary of Thermal Injury

Human cells that are exposed briefly to relatively high temperatures respond in proportion to the amount of thermal injury they absorb. Mild hyperthermia may result in

apoptosis, but severe hypothermia results in cell death that does not allow for a cellular response and is accidental in nature. Mild thermal injury kills a smaller number of cells and leads to an acceleration of repair in injured tissue through induction of the hsp response. Sublethal exposures produce hsp and cell signals such as IL-8 that call in neutrophils that may themselves exacerbate injury if over stimulated. Higher temperature exposures for longer periods of time cause cell death. Very high temperatures cause a form of instant fixation or protein coagulation that cannot be repaired by any cellular response.

Human Retinal Pigment Epithelial Cells (ARPE-19)

The retinal pigment epithelium (RPE) plays a critical role in the development and maintenance of adjacent photoreceptors in the vertebrate retina. It is highly pigmented in vivo, making it an important target for laser eye injury because laser energy is obtained absorbed and dissipated in this cell type. It forms part of the blood retina barrier in vivo. If this cell type fails to maintain an appropriate environment within the eye, the result is rapid disorganization of the retina.

The ARPE cell line exhibits a normal karyology, and forms polarized epithelial monolayers on porous filter supports that are polarized with respect transport. RPE-specific markers CRALBP and RPE65 are transcribed as determined by Northern analysis and these proteins are translated into protein as determined by Western blotting (Dunn, Aotaki-Keen, Putkey, & Hjelmeland, 1996; Dunn et al., 1998). This cell line therefore has structural and functional properties characteristic of RPE cells in vivo, making it a reasonable model for study of retinal pigment epithelium physiology.

Thermal Injury and Gene Expression Analysis

Recent developments in gene expression profiling with macro- and microarrays have greatly increased the efficiency of gene expression analysis (Gerhold et al., 2001; Joos, Eryuksel, & Brutsche, 2003; J. Li, Pankratz, & Johnson, 2002; Nadon & Shoemaker, 2002). Arrays allow for the parallel analysis of many genes from the same sample, revealing the complex interplay of genes invoked at any given time. Stock cultures of ARPE-19 cells[1] derived from human retinal pigment epithelium (RPE) were cultivated as described by Dinh (Dinh et al., 2001). Initially, we examined the genetic response of ARPE-19 for alterations in 588 known genes to thermal injury (Dinh et al., 2001). In addition to the well-described hsp response, genes involved in a variety of other metabolic pathways were altered. Most genes exhibited no alteration in expression at 1 hour after thermal injury, and a few were mildly suppressed. By 4 hours post-treatment, however, we observed a greater than two-fold elevation for some genes, including those for proteins involved in growth arrest/repair, DNA binding, and heat shock (Dinh et al., 2001). Of particular interest were genes for the heat shock proteins hsp40, hsp70, and hsp86, the 45-kDa and 153-kDa growth arrest and DNA-damage-inducible genes, and genes for DNA binding and repair proteins. The translation of protein product from the induced mRNA expression was confirmed for hsp70 and hsp40.

Thermal Injury Experiments

For survival experiments, cells were seeded on 13-mm Thermanox coverslips in 24-well plates. For gene expression profiling, cells were cultured in 100-mm culture dishes. Thermal injury experiments were carried out after the cells reached confluence by dipping the cultured cells in Hepes-buffered saline heated to 55°C for various periods of time. To determine the optimal time of exposure for inflicting a sublethal thermal injury, preliminary experiments using

[1] ARPE-19 cells were received as a gift from Dr. Larry Hjelmeland, Department of Ophthalmology, University of California at Davis.

increasing times of heating (0–9 s) with a constant temperature of 55°C were carried out with cells grown on coverslips. The percentage of cells viable at 24 hours post-heating was determined for each experiment, using a mitochondrial redox activity indicator. The optimal time and temperature for producing a 25% decrease in redox activity was chosen. Although this level of injury did not lead to morphological changes in the cells, it produced a decline in metabolic activity and the induction of a genetic response that triggers early response and stress response genes.

Gene Expression Analysis with Arrays

For gene expression profiling, cells seeded in 100-mm petri dishes were dipped for 3 seconds and then returned to medium and the incubator for an appropriate recovery period. RNA and protein were isolated from cells at 1 hour, 4 hours, 8 hours, and 24 hours after heating. Control cells were treated in a manner identical to that for heated cells, excluding the heating step, and were isolated concurrently with the 1-hour and 24-hour thermally injured cells. In preliminary experiments (data not shown), control cells were isolated at every time point until the reproducibility of control samples between time points was verified. The gene expression experiment was repeated five times.

Cell viability after thermal injury was assessed using Alamar Blue (Biosource International, Camarillo, CA), (Figure 12) which is converted to a fluorescing compound in amounts proportional to the number of viable cells (Ahmed, Gogal, & Walsh, 1994). ^{33}P labeled targets derived from thermally injured ARPE-19 RNA were hybridized to Atlas human cDNA expression arrays (no. 7740-1; Clontech, Palo Alto, CA). Following exposure to the phosphor-imaging screens (1–4 days), the distribution and intensity of radioactivity were determined by scanning the screens with a phosphor imager (Cyclone, Packard Instruments). The images were

digitally acquired using OptiQuant software (v. 3.0, Packard Instrument), and the autoradiographs were analyzed for changes in gene expression using AtlasImage software (v. 1.5, Clontech).

⇒FIGURE 12

For analysis of gene expression, the ratio of the integrated optical density (IOD) for each cDNA target was compared between heated and control cells. Cluster analysis was then performed with software originally from Stanford University, courtesy of Dr. Michael Eisen (http://rana.lbl.gov/EisenSoftware.htm) (Eisen, Spellman, Brown, & Botstein, 1998). To determine significance, two separate array membranes were hybridized with the same control sample. The average and standard deviation of IOD ratios between the two were then used to indicate the level of difference necessary to claim a significant difference. A scatter plot of the two control IOD values illustrates the distribution along a line with the slope of the average intensity (Figure 13). With an average and standard deviation of 1.14 ± 0.32, an IOD ratio of at least 2.10 represents a significant difference ($P = 0.01$).

⇒FIGURE 13

The genetic response of human cells to sublethal thermal injury was initially assessed by gene expression profiling using macroarrays containing 588 complementary probes (Dinh et al., 2001). To determine a time and temperature for production of a reproducible sublethal injury, cell survival was assessed at a temperature of 55°C (Figure 13). At this temperature, increased time of exposure (0–9 s) to heated saline caused a linear decrease in mitochondrial redox capacity, as measured by the Alamar Blue assay at 24 hours. By 48 hours after heating, preliminary data (not shown) indicated that cells heated to 55°C for 3 seconds had viability comparable to controls. Visual inspection by phase-contrast microscopy of the cells at 24 hours

post-treatment showed that heated cells exhibited the same morphological characteristics as control cells until they have been heated for at least 6 seconds. At 6 seconds and beyond, the cells began rounding up and detaching from the plate or coverslip surface. At the chosen exposure duration of 3 seconds, there was no morphologically observable injury and mRNA was not degraded; however, there was approximately a 25% reduction in Alamar Blue conversion, and alterations in gene expression were demonstrable. The further reduction in Alamar Blue with longer heating times demonstrated the relationship between mitochondrial activity and cell viability (Larson, Doughman, Gregerson, & Obritsch, 1997). Since we used this assay as a marker of the overall damage caused by heating, it was important to account for both decreases in cell number and reduced redox capacity from injured cells (Ahmed et al., 1994; Gogal, Ahmed, & Larsen, 1997; Zhi-Jun, Sriranganathan, Vaught, Arastu, & Ahmed, 1997).

Gene expression analysis indicated that at 1 hour, 4 hours, 8 hours, and 24 hours following thermal injury (3 seconds at 55°C), about one-fifth of the genes on the membrane exhibited a significant elevation or depression in expression ($\geq$ 2-fold) by 4 hours after treatment. Genes for hsps were up-regulated, as were genes for transcription factors, growth regulation, and DNA repair. Temporal relationships were assessed by cluster analysis (Figure 13). The induction of gene expression following thermal injury involves a number of genes not previously identified as related to the stress response.

Herbimycin A-Induced Cytoprotection

In normal human epidermal keratinocytes, a short exposure to sublethal injury was cytoprotective (Figure 11) against a subsequent lethal injury. Based on this observation and due to the impracticality of using heat for induction (the core body temperature must be raised to 43°C for at least 15 minutes), we looked for drugs that might provide cytoprotection. A number

of drugs have been reported to have some enhancing or inducing influence on hsp production (Amici, Palamara, & Santoro, 1993; Brown & Rush, 1984; G. C. Li, 1983; Morris, Cumming, Latchman, & Yellon, 1996; Ohno, Fukushima, Fujiwara, & Narumiya, 1988; Rossi & Santoro, 1995). Murakami (Hegde, Zuo, Voellmy, & Welch, 1995) first reported that the antibiotic herbimycin A stimulated the heat shock response and this has been confirmed by others (Briant, Ohan, & Heikkila, 1997; Conde, Lau, Dillmann, & Mestril, 1997; Morris et al., 1996). To date, the benzoquoinoid anasamycin antibiotics, herbimycin A (HA), and geldanamycin and its derivative 17-(allylamino)-17-demethoxygeldanamycin have proven to be the most cytoprotective against thermal injury.

HA was first investigated in our laboratory for its ability to increase survival of a human cell line (ARPE-19) following thermal injury. Its effect on transcriptional activity was also assessed with cDNA arrays to provide new targets for cytoprotection. Alamar Blue was used to assess cell viability 24 hours after heating ARPE-19 cells in a HEPES buffered saline bath for 6-8 seconds at 55°C. As previously demonstrated, dipping the cells attached to the coverslip into a saline bath produced a uniform injury across the cell layer with predictable survivability as a function of exposure time and temperature (Dinh et al., 2001). For the ARPE cells, a temperature of 55°C for 8 seconds resulted in a reproducible thermal injury which, when left untreated, reduced cell viability to 50% compared to unheated controls (Figure 14). Pretreatment with at least 0.75 g/ml HA for six hours prior to injury significantly increased the fraction of cells surviving an 8-second exposure by up to 50% compared to untreated controls (Figure 14).

⇒FIGURE 14

Pretreatment with HA for 1 hour followed by a delay of 4 hours before heating increased cell survival in a dose-dependent manner for thermally injured cells (Figure 15). For all durations

of heating, cell survival was notably increased by pretreatment with concentrations of at least 0.75 μg/ml HA. Under the most severe thermal insult at 8 seconds, cell survival was significantly increased by nearly all concentrations of HA (0.10 μg/ml with $P<0.05$ and 0.50-1.00 μg/ml with $P<0.01$). HA increased cell survival from 15% to 50% compared to untreated cells (Figure 14).

Gene Expression Analysis of HA-Induced Cytoprotection

To better understand the basis for HA cytoprotection and to acquire information about potential cytotoxic effects of HA, we performed gene expression analysis with cDNA arrays after treatment with HA for 1 hour, 4 hours, or 24 hours. Over the 24-hour treatment period, 1.00 μg/ml HA significantly altered transcriptional regulation of 307 of the 1176 genes on the Atlas™ Human 1.2 cDNA array interrogating 1188 genes. Approximately one-fifth of the genes that were significantly elevated by 1 hour remained elevated at 4 hours or 24 hours, although typically to a lesser extent. Transcription of this set of genes peaked at 1 hour and generally decreased over 24 hours to lower or control levels. Some of the most significantly elevated genes at 1 hour included transcription factor 3, jun D proto-oncogene, cyclin G associated kinase, endoglin, interferon regulating factor 7, macrophage receptor, and heat shock transcription factor 4. The only significantly suppressed genes were DiGeorge syndrome critical region gene DGSI, member 3 of the potassium channel subfamily K, and mucosal vascular addressin cell-adhesion molecule 1.

⇒FIGURE 15

Treatment with HA for just 4 hours caused alterations in just 47 genes, of which 19 were newly elevated and had not been significantly altered at 1 hour. Most of these genes returned to control levels by 24 hours, although several were still significantly elevated or suppressed at 24

hours. Some newly up-regulated genes at 4 hours included runt-related transcription factor 3, hsp90, hsp70, and plasminogen activator inhibitor. After 24 hours of HA treatment, 57 genes were also significantly altered. Approximately one-third of these genes were newly expressed and also predominantly suppressed. The temporal pattern of expression for all altered genes was more easily evaluated through clustering. Analysis of the most highly regulated subset of genes would suggest which cellular functions are most important for the repair of injured cells. The dendrogram organized the subset of genes (altered by 3-fold) into groupings that were regulated in a similar manner over the 24-hour time course (Figure 14).

Furthermore, gene expression profiling following HA treatment demonstrated that the most highly elevated genes included growth factors and transcription factors, while prominently suppressed genes included transcription factors and kinases (Figure 15). These results suggest that cytoprotection may be due to the contribution of the products of a significant number of genes in addition to the classic stress response genes. Modulation of these genes might induce thermotolerance and amelioration of thermal injury.

Although most genes exhibited no alteration in expression by 1 hour after thermal injury, a few were mildly suppressed. By 4 hours post-treatment, a greater than two-fold elevation was observed for some genes, including growth arrest/repair, DNA binding, and heat shock and other stress response proteins. Of particular importance for their known role in a survivable thermal injury are the heat shock proteins hsp40, hsp70, and hsp86, the 45-kDa and 153-kDa growth arrest and DNA-damage-inducible genes, and genes for DNA-binding and repair proteins. The translation of protein product from the induced mRNA expression was confirmed for hsp70 and hsp40. By observing the gene response to thermal stress in an in vitro model, we may better understand the benefits and targets of drug treatment for secondary damage associated with

thermal injury. Gene expression profiling may be applied to drug discovery, development, and evaluation by defining cell or tissue responses and assessing drug mechanisms of action. As we gather more knowledge of how and why genes function, we can use this knowledge to systematically find targets and complementary drugs for enhancing treatment.

Since the induction of hsp cytoprotection is through gene transcription and translation, cDNA arrays were used to identify additional genes involved in cytoprotection or cytotoxicity of HA. Gene expression profiling indicated that in addition to the induction of the classic heat shock genes (i.e., hsp90, hsp70), transcription for a variety of other genes encoding growth factors and transcription factors were up-regulated and may be involved in protection by HA. Therefore, HA likely exerts some of its cytoprotection through other mechanisms. By understanding how altered gene expression is involved in specific pathways of induction and by identifying the resulting downstream changes in cellular function and expression, additional mechanisms of protection may be elucidated.

Verification of hsp Production

Heat shock protein levels were measured over a 24-hour period following HA treatment of uninjured cells to determine the relationship between message and protein appearance. ARPE-19 cells were treated with 1.00 μg/ml HA for 1 hour, 4 hours, 8 hours, and 24 hours and were then evaluated over a 24-hour time period for production of hsps (Dinh et al., 2002)

Thermal injury experiments verified that ARPE-19 cells pretreated with 0.75-1.00 g/ml HA were protected against lethal thermal insult at 55°C. Gene expression profiling of ARPE-19 cells treated with HA revealed a significant up-regulation of mRNA for hsp70 and hsp90. Western blotting corroborated protein production for hsp40 and hsp70. The mRNA and protein results also indicated that there was a lag time between up-regulation in transcription and

production of hsp70 product. It is therefore conceivable that with a longer response time before thermal injury, allowing for greater hsp70 protein production, it may be possible to obtain an improved protective response. The observed significant increase in cell survival suggests that HA confers cytoprotection against thermal injury, but the low levels of hsp70 production (2.1-fold increase) suggest that other genes may also play a role in providing protection. Many other genes were up-regulated by more than 3-fold. It remains to be determined if post-injury treatment may provide any cytoprotection.

Recent studies also suggest that HA may indirectly induce hsps by acting on other kinases or phosphatases that then directly phosphorylate and activate heat shock factor1 (HSF1) (Trump et al., 1997). It has been shown that activation of HSF1 was possible even though it was not phosphorylated at any tyrosine residues (Gabai, 2002 #2069). This finding implied that phosphorylation and de-phosphorylation take place at the tyrosine residue of a mediating kinase or phosphatase (Keyse & Emslie, 1992). In further support of this theory, a study using genistein, a tyrosine kinase inhibitor employing a different mode of action, was unable to induce hsps even when used at doses that inhibit tyrosine kinase activity (Morris et al., 1996). It has also been suggested that HA may play a role in the inhibition of IκB phosphorylation by preventing the activation and nuclear translocation of NFκB. These findings therefore suggest a possible relationship between HA induction of hsps and the inhibition of NFκB.

Gene expression profiling of HA treated cells suggested that cytoprotection from thermal insult might also be due to the regulation of transcription factors and inhibition of NFκB activation. While Western blotting confirmed the production of hsp40 and hsp70, the cDNA arrays exemplified the presence of mRNA for several hsps in addition to several hundred additional genes. These results suggest that the effectiveness of HA in providing cytoprotection

is likely attributable to the intricate relationship between hsp induction and the modulation of kinase activity and signal transduction.

Microarray technology is providing a new perspective on the development of drugs for the treatment of disease. From the therapeutic perspective, little is known about cellular response to thermal injury. What we need to know is how to rescue thermally injured tissue so that the effects of severe trauma and convalescence time can be reduced. Since the wound healing response after thermal injury is mediated by sub-lethally injured and surviving cells, the findings reported here may have application in the development of treatments for small and blinding focal thermal injuries such as those that occur after accidental laser exposure to the retinal macula (Humphrey, Parker, Chu, & Constable, 1993). A better understanding of the hsp response may improve clinical applications of laser effects that are used in the treatment of photo-aged skin, corneal shape remodeling, cataract removal, transmyocardial revascularization, surgical excisions, hair removal, and pan-retinal photocoagulation in diabetic retinopathy. The determination that genes other than the hsps are induced by HA suggests that alternate pathways may exist for therapeutic development of cytoprotectants.

Comparability of CO_2 Laser Injury to Thermal Injury

The initial problem of the Gaussian distribution of energy across the surface of a monolayer has recently been overcome by using a scanning system that quickly sweeps across the cell layer delivering a uniform amount of energy (Walker et al., 2007) (Figure 16). We have recently demonstrated a uniform distribution of hsps across a cell layer of ARPE-19 cells scanned with such a system using the carbon dioxide laser. We are now in the process of determining the temperature to which the monolayer was raised and we will perform gene expression analysis on cells so treated. Cells were grown to 95% confluency (if cell confluency

was either less than 80% or greater than 100%, CO_2 laser injury was not reproducible) in 24-well Costar multiplates (Corning). The carbon dioxide laser system (10.6 um, 2.0 W/cm^2, nominal 2.0 mm diameter spot size, and peak intensity of 64 W/cm^2 with a Gaussian beam profile) was aligned and the beam was directed through steering optics to a set of scanning X-Y mirrors that directed the beam through a small limiting aperture (Figure 16). An alignment target beneath the aperture was used to align the well bottoms for exposure. The well bottoms of the 24-well plate had a small clearance from the target surface, eliminating any heat conduction or cooling phenomena. The beam scan path had a 90% scan area overlap and the calculated exposure time was approximately 700ms/10 um^2.

⇒FIGURE 16

For exposure, cells were irradiated with a raster pattern produced by scanning the CO_2 laser beam controlled by a pair of angular rotating mirrors mounted with axes perpendicular to one another. The computer controlled raster scan was varied in total scan time to adjust the dwell time of the laser beam on the cells. Since the beam diameter was larger than the individual cells, each cell was calculated to receive a sequence of ~13 exposures of varying thermal intensity as the beam was repetitively scanned across it. In our experiments, total scan times ranged from 8 seconds to 15 seconds, corresponding to time-averaged irradiant energy doses ranging from 10.4J/cm^2 to 19.5J/cm^2 averaged over the whole monolayer of cells.

Preliminary temperature measurements were made during the exposure with an infrared thermometer probe directed to wells that had the well wall reduced to a height of 1.0 mm. Cells at 95% confluency and with medium removed were then evaluated for a rise in temperature on exposure to the laser.

For irradiation, the volume of media (DMEM) was reduced to 500 ul/well and was carefully pipeted out of the well bottom with a micro-tip pipet in a manner consistent with slow sheeting of the water film from the cell surfaces to ensure nearly complete and reproducible removal. A critical factor for reproducibility required almost complete removal of the medium as water is the main absorber at 10.6 um. Next, the well bottom was aligned with the target and the scanning sequence was activated by computer control. After each exposure, media was replaced into each well. Approximately 30 minutes later, media was replaced with 1.0 ml of fresh DMEM and plates were returned to a 37°C incubator (95% O_2/5% CO_2). Cells were followed by morphology assessment, hsp70 immunostaining, gel electrophoresis, Western blotting, and cell viability assays. As far as we were able to ascertain by biochemical and viability data, the CO_2 laser produced an injury that was comparable to that produced by dipping cells in heated medium. Further, a 13 second CO_2 laser exposure at 2.0 W/cm^2 produced morphologic injury similar to dipping cells in heated medium for 9 seconds at 55° C, although temperature measurements need to confirm peak temperatures attained.

HA-Induced Cytoprotection of Laser Injury

Figure 17 shows phase contrast images of cells exposed to CO_2 laser (2.0W/cm^2 scanned beam over a 16 x 16 mm field for 13 s) without (a) and with (b) a 6-hour pretreatment with HA (1 μg/ml). Cell survival data based on phase contrast cell count assessments show that both HA and 3 seconds of heat pretreatment were highly effective in promoting cell survival against CO_2 laser irradiation (Figure 17), similar to the cytoprotection demonstrated in the lethal heated saline injury model (Figure 14).

⇒FIGURE 17

Summary, Recommendations, and Future Directions

We have demonstrated that human cells undergo a variety of responses to thermal injury depending on time and temperature of exposure and that the CO_2 laser produces injury that is predominantly thermal. Of the estimated 20,000-25,000 genes that constitute the human genome, only a small fraction of these genes have been evaluated for their role in this response. The possibility of interrogating the entire genome on a single DNA chip was realized in 2003 (Affymetrix, Inc., Santa Clara, CA) and should be applied to this question. By examining this response as a function of time, it becomes possible to distinguish early, causative actions and downstream effects. After what is initially a descriptive or ontological effort, broad hypothesis testing can begin with some assurance that appropriate targets have been identified. Functional genomic techniques must then be applied to understand what role a gene or set of genes may play in a particular response. A better understanding of the genetic response to thermal injury will yield new insights and new targets for therapy.

We have begun to develop drug treatments based on this understanding and have demonstrated that if administered as pretreatments, bensaquinoid ansamycin antibiotics are potent cytoprotectants against thermal and CO_2 laser injury. This advance was the result of identifying the correct nature of the injury and looking for ways to protect against it by screening for drugs that have been reported to induce the heat shock response. Identification of other response pathways may lead to additional drugs that can be administered after injury.

Finally, if the application of the scanning technique that we have applied to study thermal effects of lasers can be applied to laser systems that produce photochemical and photomechanical injury, a better understanding of these types of injuries may lead to newer and highly selective methods of treatment. This screening technique provides a very economical lead

discovery approach that has proven effective and is commonly employed by large pharmaceutical companies.

REFERENCES

Ahmed, S. A., Gogal, R. M., Jr., & Walsh, J. E. (1994). A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: an alternative to [3H]thymidine incorporation assay. *J Immunol Methods, 170*(2), 211-224.

Amici, C., Palamara, A. T., & Santoro, M. G. (1993). Induction of thermotolerance by prostaglandin A in human cells. *Experimental Cell Research, 207*(2), 230-234.

Anderson, R. R. (2000). Lasers in dermatology--a critical update. *J Dermatol, 27*(11), 700-705.

Ash, K., Lord, J., Newman, J., & McDaniel, D. H. (1999). Hair removal using a long-pulsed alexandrite laser. *Dermatol Clin, 17*(2), 387-399, ix.

Bargman, H. (1999). Point-counterpoint: laser hair removal. *J Cutan Med Surg, 3*(5), 241.

Barrett, S. F., Wright, C. H., Oberg, E. D., Rockwell, B. A., Cain, C., Rylander, H. G., 3rd, et al. (1996). Development of an integrated automated retinal surgical laser system. *Biomed Sci Instrum, 32*, 215-224.

Baugh, W. P., Trafeli, J. P., Barnette, D. J., Jr., & Ross, E. V. (2001). Hair reduction using a scanning 800 nm diode laser. *Dermatol Surg, 27*(4), 358-364.

Bencini, P. L., Luci, A., Galimberti, M., & Ferranti, G. (1999). Long-term epilation with long-pulsed neodimium:YAG laser. *Dermatol Surg, 25*(3), 175-178.

Bonfoco, E., Krainc, D., Ankarcrona, M., Nicotera, P., & Lipton, S. A. (1995). Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with N-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. *Proc Natl Acad Sci U S A, 92*(16), 7162-7166.

Bowman, P. D., Schuschereba, S. T., Lawlor, D. F., Gilligan, G. R., Mata, J. R., & DeBaere, D. R. (1997). Survival of human epidermal keratinocytes after short-duration high temperature: synthesis of HSP70 and IL-8. *American Journal of Physiology, 272*(6 Pt 1), C1988-1994.

Briant, D., Ohan, N., & Heikkila, J. J. (1997). Effect of herbimycin A on hsp30 and hsp70 heat shock protein gene expression in Xenopus cultured cells. *Biochem Cell Biol, 75*(6), 777-782.

Brown, I. R., & Rush, S. J. (1984). Induction of a 'stress' protein in intact mammalian organs after the intravenous administration of sodium arsenite. *Biochem Biophys Res Commun, 120*(1), 150-155.

Calabrese, E. J., Bachmann, K. A., Bailer, A. J., Bolger, P. M., Borak, J., Cai, L., et al. (2007). Biological stress response terminology: Integrating the concepts of adaptive response and preconditioning stress within a hormetic dose-response framework. *Toxicol Appl Pharmacol, 222*(1), 122-128.

Cervos-Navarro, J., & Schubert, T. E. (1996). Pitfalls in the evaluation of apoptosis using TUNEL. *Brain Pathol, 6*(3), 347-348.

Charriaut-Marlangue, C., & Ben-Ari, Y. (1995). A cautionary note on the use of the TUNEL stain to determine apoptosis. *Neuroreport, 7*(1), 61-64.

Chiarelli, T. G., & Muglia, J. J. (2001). The age of enlightenment: current uses of lasers in dermatology. *Med Health R I, 84*(9), 288-291.

Conde, A. G., Lau, S. S., Dillmann, W. H., & Mestril, R. (1997). Induction of heat shock proteins by tyrosine kinase inhibitors in rat cardiomyocytes and myogenic cells confers protection against simulated ischemia. *J Mol Cell Cardiol, 29*(7), 1927-1938.

de Torres, C., Munell, F., Ferrer, I., Reventos, J., & Macaya, A. (1997). Identification of necrotic cell death by the TUNEL assay in the hypoxic-ischemic neonatal rat brain. *Neurosci Lett, 230*(1), 1-4.

Dewey, W. C. (1979). Recommendations for future research in hyperthermia at the molecular, cellular, and animal levels. *Cancer Research, 39*(6 Pt 2), 2330-2331.

Dewey, W. C. (1984). Interaction of heat with radiation and chemotherapy. *Cancer Research, 44*(10 Suppl), 4714s-4720s.

Dinh, H. K., Stavchansky, S., Schuschereba, S. T., Stuck, B. E., & Bowman, P. D. (2002). Cytoprotection against thermal injury: evaluation of herbimycin A by cell viability and cDNA arrays. *Pharmacogenomics J, 2*(5), 318-326.

Dinh, H. K., Zhao, B., Schuschereba, S. T., Merrill, G., & Bowman, P. D. (2001). Gene expression profiling of the response to thermal injury in human cells. *Physiol Genomics, 7*(1), 3-13.

Dunn, K. C., Aotaki-Keen, A. E., Putkey, F. R., & Hjelmeland, L. M. (1996). ARPE-19, a human retinal pigment epithelial cell line with differentiated properties. *Experimental Eye Research, 62*(2), 155-169.

Dunn, K. C., Marmorstein, A. D., Bonilha, V. L., Rodriguez-Boulan, E., Giordano, F., & Hjelmeland, L. M. (1998). Use of the ARPE-19 cell line as a model of RPE polarity: basolateral secretion of FGF5. *Investigative Ophthalmology & Visual Science, 39*(13), 2744-2749.

Dypbukt, J. M., Ankarcrona, M., Burkitt, M., Sjoholm, A., Strom, K., Orrenius, S., et al. (1994). Different prooxidant levels stimulate growth, trigger apoptosis, or produce necrosis of insulin-secreting RINm5F cells. The role of intracellular polyamines. *J Biol Chem, 269*(48), 30553-30560.

Eisen, M. B., Spellman, P. T., Brown, P. O., & Botstein, D. (1998). Cluster analysis and display of genome-wide expression patterns. *Proceedings of the National Academy of Sciences of the United States of America, 95*(25), 14863-14868.

Ferrando, R. E., Schuschereba, S. T., Quong, J. A., & Bowman, P. D. (1995). Carbon dioxide laser induction of heat shock protein 70 synthesis: comparision with high temperature treatment. *LASER IN MEDICAL SCIENCES10*, 207-212.

Garner, W. L., Rodriguez, J. L., Miller, C. G., Till, G. O., Rees, R. S., Smith, D. J., et al. (1994). Acute skin injury releases neutrophil chemoattractants. *Surg, 116*, 42-48.

Gavrieli, Y., Sherman, Y., & Ben-Sasson, S. A. (1992). Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J Cell Biol, 119*(3), 493-501.

Gerhold, D., Lu, M., Xu, J., Austin, C., Caskey, C. T., & Rushmore, T. (2001). Monitoring expression of genes involved in drug metabolism and toxicology using DNA microarrays. *Physiol Genomics, 5*(4), 161-170.

Gogal, R. M., Jr., Ahmed, S. A., & Larsen, C. T. (1997). Analysis of avian lymphocyte proliferation by a new, simple, nonradioactive assay (lympho-pro). *Avian Dis, 41*(3), 714-725.

Grasl-Kraupp, B., Ruttkay-Nedecky, B., Koudelka, H., Bukowska, K., Bursch, W., & Schulte-Hermann, R. (1995). In situ detection of fragmented DNA (TUNEL assay) fails to discriminate among apoptosis, necrosis, and autolytic cell death: a cautionary note. *Hepatology, 21*(5), 1465-1468.

Hegde, R. S., Zuo, J., Voellmy, R., & Welch, W. J. (1995). Short circuiting stress protein expression via a tyrosine kinase inhibitor, herbimycin A. *Journal of Cellular Physiology, 165*(1), 186-200.

Henle, K. J., & Warters, R. L. (1982). Heat protection by glycerol in vitro. *Cancer Res, 42*(6), 2171-2176.

Henseleit, U., Zhang, J., Wanner, R., Haase, I., Kolde, G., & Rosenbach, T. (1997). Role of p53 in UVB-induced apoptosis in human HaCaT keratinocytes. *J Invest Dermatol, 109*(6), 722-727.

Hirsch, T., Marzo, I., & Kroemer, G. (1997). Role of the mitochondrial permeability transition pore in apoptosis. *Biosci Rep, 17*(1), 67-76.

Humphrey, M. F., Parker, C., Chu, Y., & Constable, I. J. (1993). Transient preservation of photoreceptors on the flanks of argon laser lesions in the RCS rat. *Current Eye Research, 12*(4), 367-372.

Joos, L., Eryuksel, E., & Brutsche, M. H. (2003). Functional genomics and gene microarrays--the use in research and clinical medicine. *Swiss Med Wkly, 133*(3-4), 31-38.

Keyse, S. M., & Emslie, E. A. (1992). Oxidative stress and heat shock induce a human gene encoding a protein-tyrosine phosphatase. *Nature, 359*(6396), 644-647.

Klavuhn, K. G., & Green, D. (2002). Importance of cutaneous cooling during photothermal epilation: theoretical and practical considerations. *Lasers Surg Med, 31*(2), 97-105.

Kroemer, G., Zamzami, N., & Susin, S. A. (1997). Mitochondrial control of apoptosis. *Immunol Today, 18*(1), 44-51.

Kruuv, J., Glofcheski, D., Cheng, K. H., Campbell, S. D., Al-Qysi, H. M., Nolan, W. T., et al. (1983). Factors influencing survival and growth of mammalian cells exposed to hypothermia. I. Effects of temperature and membrane lipid perturbers. *Journal of Cellular Physiology, 115*(2), 179-185.

Larson, E. M., Doughman, D. J., Gregerson, D. S., & Obritsch, W. F. (1997). A new, simple, nonradioactive, nontoxic in vitro assay to monitor corneal endothelial cell viability. *Investigative Ophthalmology & Visual Science, 38*(10), 1929-1933.

Lepock, J. R., & Kruuv, J. (1980). Thermotolerance as a possible cause of the critical temperature at 43 degrees in mammalian cells. *Cancer Research, 40*(12), 4485-4488.

Levinson, W., Opperman, H., & Jackson, J. (1980). Transition series metals and sulfhydryl reagents induce the synthesis of four proteins in eukaryotic cells. *Biochemica et Biophysica Acta, 606*, 170-180.

Li, C. G. (1982). Induction of thermotolerance and enhanced heat shock protein synthesis in chinese hamster fibroblasts by sodium arsenite and by ethanol. *Journal of Cellular Physiology, 115*, 116-122.

Li, G. C. (1983). Induction of thermotolerance and enhanced heat shock protein synthesis in Chinese hamster fibroblasts by sodium arsenite and by ethanol. *Journal of Cellular Physiology, 115*(2), 116-122.

Li, J., Pankratz, M., & Johnson, J. A. (2002). Differential gene expression patterns revealed by oligonucleotide versus long cDNA arrays. *Toxicol Sci, 69*(2), 383-390.

Lindquist, S. (1986). The heat shock response. *Annual Reviews in Biochemistry, 55*, 1151-1191.

Lindquist, S., & Craig, E. A. (1988). The heat-shock proteins. *Annu Rev Genet.*

Lotem, J., Peled-Kamar, M., Groner, Y., & Sachs, L. (1996). Cellular oxidative stress and the control of apoptosis by wild-type p53, cytotoxic compounds, and cytokines. *Proc Natl Acad Sci U S A, 93*(17), 9166-9171.

Lotem, J., & Sachs, L. (1996). Differential suppression by protease inhibitors and cytokines of apoptosis induced by wild-type p53 and cytotoxic agents. *Proc Natl Acad Sci U S A, 93*(22), 12507-12512.

Macho, A., Decaudin, D., Castedo, M., Hirsch, T., Susin, S. A., Zamzami, N., et al. (1996). Chloromethyl-X-Rosamine is an aldehyde-fixable potential-sensitive fluorochrome for the detection of early apoptosis. *Cytometry, 25*(4), 333-340.

Mackey, M. A., Morgan, W. F., & Dewey, W. C. (1988). Nuclear fragmentation and premature chromosome condensation induced by heat shock in S-phase Chinese hamster ovary cells. *Cancer Research, 48*(22), 6478-6483.

Magal, S. S., Jackman, A., Pei, X. F., Schlegel, R., & Sherman, L. (1998). Induction of apoptosis in human keratinocytes containing mutated p53 alleles and its inhibition by both the E6 and E7 oncoproteins. *Int J Cancer, 75*(1), 96-104.

Mainster, M. A. (1986). Wavelength selection in macular photocoagulation. Tissue optics, thermal effects, and laser systems. *Ophthalmology, 93*(7), 952-958.

Mainster, M. A., Crossman, J. L., Erickson, P. J., & Heacock, G. L. (1990). Retinal laser lenses: magnification, spot size, and field of view. *Br J Ophthalmol, 74*(3), 177-179.

Mainster, M. A., White, T. J., & Allen, R. G. (1970). Spectral dependence of retinal damage produced by intense light sources. *J Opt Soc Am, 60*(6), 848-855.

Mainster, M. A., White, T. J., Tips, J. H., & Wilson, P. W. (1970). Retinal-temperature increases produced by intense light sources. *Journal of the Optical Society of America, 60*(2), 264-270.

Majno, G., & Joris, I. (1995). Apoptosis, oncosis, and necrosis. An overview of cell death. *Am J Pathol, 146*(1), 3-15.

Marchetti, P., Hirsch, T., Zamzami, N., Castedo, M., Decaudin, D., Susin, S. A., et al. (1996). Mitochondrial permeability transition triggers lymphocyte apoptosis. *J Immunol, 157*(11), 4830-4836.

Marshall, J., & Mellerio, H. J. (1968). Histology of retinal lesions produced with Q-switched lasers. *Exp Eye Res, 7*(2), 225-230.

Marshall, J., & Mellerio, J. (1967a). Histology of the formation of retinal laser lesions. *Exp Eye Res, 6*(1), 4-9.

Marshall, J., & Mellerio, J. (1967b). Pathological development of retinal laser photocoagulations. *Experimental Eye Research, 6*(4), 303-308.

Marshall, J., & Mellerio, J. (1970). Laser irradiation of retinal tissue. *Br Med Bull, 26*(2), 156-160.

Matylevitch, N. P., Schuschereba, S. T., Mata, J. R., Gilligan, G. R., Lawlor, D. F., Goodwin, C. W., et al. (1998). Apoptosis and accidental cell death in cultured human keratinocytes after thermal injury. *American Journal of Pathology, 153*(2), 567-577.

Maytin, E. V., Wimberly, J. M., & Anderson, R. R. (1990). Thermotolerance and the heat shock response in normal human keratinocytes in culture. *J Invest Dermatol, 95*(6), 635-642.

Mellerio, J., Capon, M. R., Docchio, F., Sliney, D., & Krafft, J. (1988). A new form of damage to PMMA intraocular lenses by Nd:YAG laser photodisruptors. *Eye, 2 (Pt 4)*, 376-381.

Morimoto, R. I., & Santoro, M. G. (1998). Stress-inducible responses and heat shock proteins: new pharmacologic targets for cytoprotection. *Nature Biotechnology, 16*(9), 833-838.

Moritz, A. R., & Henriques, F. C. J. (1947). Studies of thermal injury II. The relative importance of time and surface temperature in the causation of cutaneous burns. *Am J Pathol, 23*, 695-720.

Morris, S. D., Cumming, D. V., Latchman, D. S., & Yellon, D. M. (1996). Specific induction of the 70-kD heat stress proteins by the tyrosine kinase inhibitor herbimycin-A protects rat neonatal cardiomyocytes. A new pharmacological route to stress protein expression? *Journal of Clinical Investigation, 97*(3), 706-712.

Mosman, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application ot proliferation and cytotoxicity assays. *J Immunol Methods, 65*,, 55-63.

Nadon, R., & Shoemaker, J. (2002). Statistical issues with microarrays: processing and analysis. *Trends Genet, 18*(5), 265-271.

Nakamura, T., Sakai, T., & Hotchi, M. (1995). Histochemical demonstration of DNA double strand breaks by in situ 3'-tailing reaction in apoptotic endometrium. *Biotech Histochem, 70*(1), 33-39.

Nover, L. (1984). Heat shock response of eukaryotic cells. *New York Tokyo, Springer-Verlag.*

Ohno, K., Fukushima, M., Fujiwara, M., & Narumiya, S. (1988). Induction of 68,000-dalton heat shock proteins by cyclopentonone prostaglandins. *The Journal of Biological Chemistry, 36*, 19764-19770.

Papp, E., Nardai, G., Soti, C., & Csermely, P. (2003). Molecular chaperones, stress proteins and redox homeostasis. *Biofactors, 17*(1-4), 249-257.

Parcellier, A., Gurbuxani, S., Schmitt, E., Solary, E., & Garrido, C. (2003). Heat shock proteins, cellular chaperones that modulate mitochondrial cell death pathways. *Biochem Biophys Res Commun, 304*(3), 505-512.

Petit, P. X., Zamzami, N., Vayssiere, J. L., Mignotte, B., Kroemer, G., & Castedo, M. (1997). Implication of mitochondria in apoptosis. *Mol Cell Biochem, 174*(1-2), 185-188.

Polakowska, R. R., Piacentini, M., Bartlett, R., Goldsmith, L. A., & Haake, A. R. (1994). Apoptosis in human skin development: morphogenesis, periderm, and stem cells. *Dev Dyn, 199*(3), 176-188.

Pruitt, B., Goodwin, CW, Pruitt, SK. (1991). *Burns: including cold, chemical, and electrical injuries.* Philadelphia, PA: W.B. Saunders Co.

Raaphorst, G. P., Feeley, M. M., Chu, G. L., & Dewey, W. C. (1993). A comparison of the enhancement of radiation sensitivity and DNA polymerase inactivation by hyperthermia in human glioma cells. *Radiation Research, 134*(3), 331-336.

Rice, G. C., Gray, J. W., Dean, P. N., & Dewey, W. C. (1984). Fluorescence-activated cell sorting analysis of the induction and expression of acute thermal tolerance within the cell cycle. *Cancer Research, 44*(6), 2368-2376.

Rice, G. C., Gray, J. W., & Dewey, W. C. (1984). Cycle progression and division of viable and nonviable Chinese hamster ovary cells following acute hyperthermia and their relationship to thermal tolerance decay. *Cancer Research, 44*(5), 1802-1808.

Ritossa, F. M. (1962). A new puffing pattern induced by a temperature shock and DNP in Drosophila. *Experimenta, 18*, 571-573.

Rockwell, J. R. (1994). Laser accidents: reviewing thirty years of incidents: what are the concerns-old and new? *J Laser Applications, 6*, 203-211.

Rossi, A., & Santoro, M. G. (1995). Induction by prostaglandin A1 of haem oxygenase in myoblastic cells: an effect independent of expression of the 70 kDa heat shock protein. *Biochemical Journal, 308*(Pt 2), 455-463.

Sapareto, S. A., & Dewey, W. C. (1984). Thermal dose determination in cancer therapy. *International Journal of Radiation Oncology, Biology, Physics, 10*(6), 787-800.

Scannapieco, A. C., Sorensen, J. G., Loeschcke, V., & Norry, F. M. (2007). Heat-induced hormesis in longevity of two sibling Drosophila species. *Biogerontology, 8*(3), 315-325.

Schirmer, K. E. (1992). Simultaneous thermal and optical breakdown mode dual laser action. *Ophthalmologica, 205*(4), 169-177.

Schuschereba, S. T., Ferrando, R. E., Stuck, B. E., Quong, J. A., Lund, D. J., & Bowman, P. D. (1994). Stimulation of heat shock protein 70 synthesis in human fibroblasts by short duration (,1 sec) carbon dioxide laser radiation. *Lasers in the Life Sciences, 6*, 27-38.

Sliney, D., & Wolbarsht, M. (1980). *Safety with lasers and other optical sources: a comprehensive handbook.* New York: Plenum Press.

Sliney, D. H. (1985). Laser-tissue interactions. *Clin Chest Med, 6*(2), 203-208.

Sliney, D. H., & Wolbarsht, M. L. (1989). Future applications of lasers in surgery and medicine: a review. *J R Soc Med, 82*(5), 293-296.

Sliney, D. H., & Wolborsht, M. L. (1980). Safety standards and measurement techniques for high intensity light sources. *Vision Res, 20*(12), 1133-1141.

Subjeck, J. R., Sciandra, J. J., & Shyy, T. T. (1985). Analysis of the expression of the two major proteins of the 70 kilodalton mammalian heat shock family. *Int J Radiat Biol Relat Stud Phys Chem Med, 47*(3), 275-284.

Trump, B. F., Berezesky, I. K., Chang, S. H., & Phelps, P. C. (1997). The pathways of cell death: oncosis, apoptosis, and necrosis. *Toxicologic Pathology, 25*(1), 82-88.

van Lookeren Campagne, M., & Gill, R. (1996). Ultrastructural morphological changes are not characteristic of apoptotic cell death following focal cerebral ischaemia in the rat. *Neuroscience Letters, 213*(2), 111-114.

Walker, K. P., 3rd, Schuschereba, S. T., Edsall, P. R., Stuck, B. E., & Bowman, P. D. (2007). Production of a uniform cellular injury by raster scanning of cells for the study of laser bioeffects. *Am J Physiol Cell Physiol, 292*(4), C1536-1542.

Welch, A. J. (1984). The thermal response of laser irradiated tissue. *IEEE Journal of Quantum Electronics, QE-20*(12), 1471-1481.

Welch, W. J., & Suhan, J. P. (1985). Morphological study of the mammalian stress response: characterization of changes in cytoplasmic organelles, cytoskeleton, and nucleoli, and appearance of intranuclear actin filaments in rat fibroblasts after heat-shock treatment. *J Cell Biol, 101*(4), 1198-1211.

Zhi-Jun, Y., Sriranganathan, N., Vaught, T., Arastu, S. K., & Ahmed, S. A. (1997). A dye-based lymphocyte proliferation assay that permits multiple immunological analyses: mRNA, cytogenetic, apoptosis, and immunophenotyping studies. *J Immunol Methods, 210*(1), 25-39.

Figure 1

Fig. 1. 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT) staining of laser impact zone. Single-spot, carbon dioxide laser exposure of an adult retinal pigment epithelial (ARPE)-19 cell monolayer (10.6- m, 4.0-mm spot size, 41.3 W/cm2 irradiance, 150 ms duration). A: 21.4 magnification showing the absence of viable cells in the center of the lesion and rounded-up cells with some formazan deposition on the edges. B: 375 magnification of MTT staining showing dead cells in the center of the lesion with no formazan (blue arrow) and viable cells on the edge (black arrow). C: 21.4 magnification of laser impact zone with in situ immunolocalization of hsp70 showing the thin zone of affected cells exhibiting hsp70 induction. D: 375 magnification of cells about the edge of the lesion showing lack of staining in center, responders (demarcated with black arrows) grading into nonresponders outside the impact zone. Scale bars 2 mm in A and C and 100 m in C and D. In studies of such lesions, only a small fraction of cells are responders, resulting in poor signal-to-noise ratios for biochemical and molecular studies.

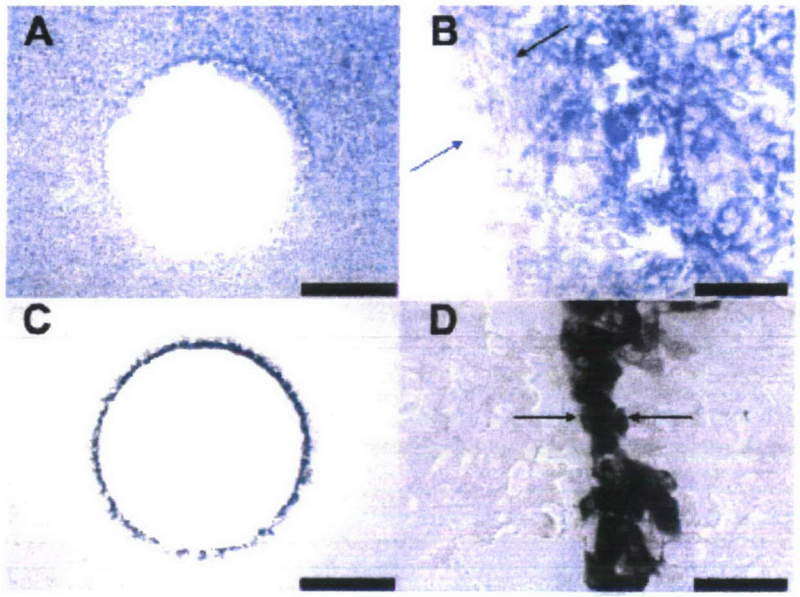


Figure 2

Survival of NHEK assessed by the MTT viability assay as a function of time after heating. Groups of 3 coverslips were exposed to a 1 second heat treatment and then assayed for viability at 1, 3 and 6 days. Y-axis is a log scale and 1 represents 100 percent survival.

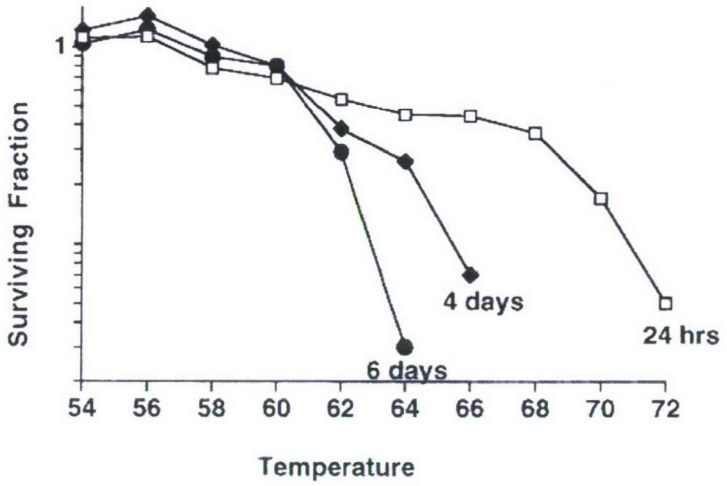


Figure 3

Arrhenius plot for inactivation of cells by heat. The y-axis is the reciprocal of the inactivation rate while the upper x-axis is the reciprocal of absolute temperature. The lower x-axis presents the temperature in degrees centigrade. Heat inactivation energy was calculated as described after Westra and Dewey

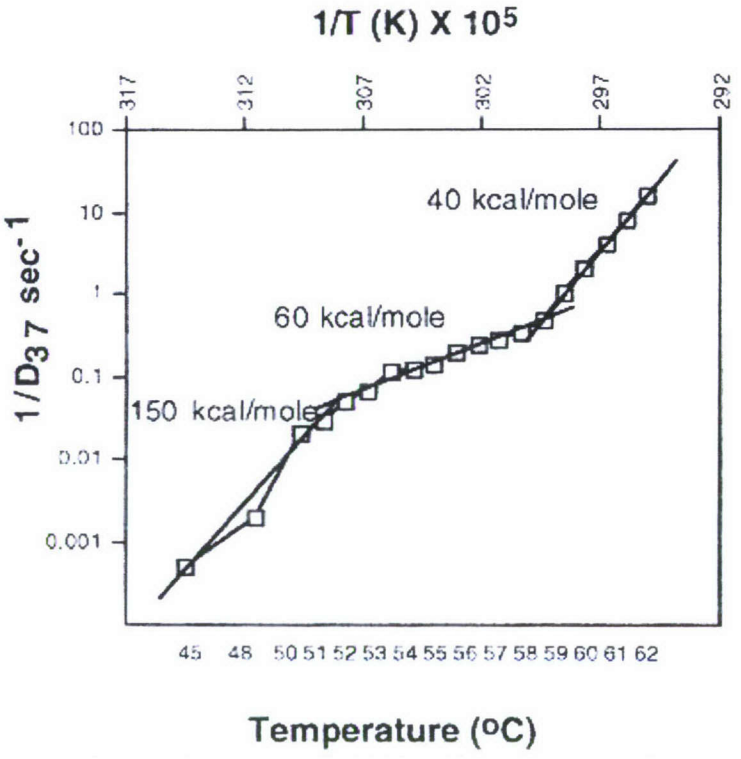


Figure 4

Anti-hsp 70 staining of immunoblots exposed to 1 or 4 seconds of elevated temperature. Integrated densitometric quantification of hsp 70 is shown below each blot. The image of each blot was captured with a video camera and framegrabber and the density of the reflicted digitized image was quantified with the NIH Image program. Bars represent increased density above control values.

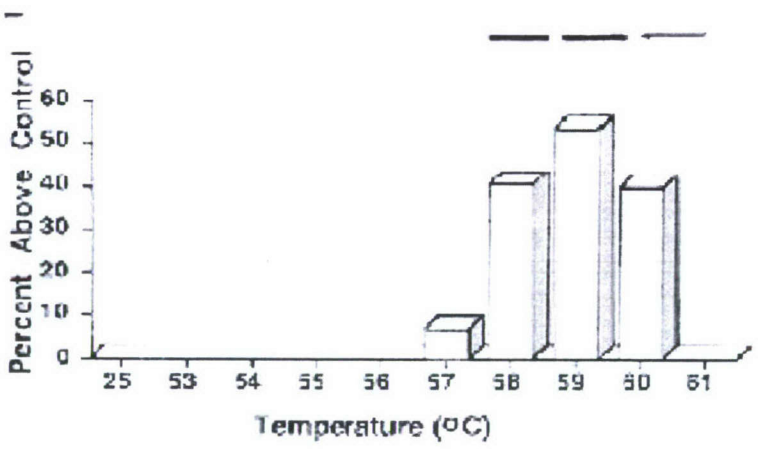


Figure 5

Release of IL-8 (pg/ml) by NHEK during the 24 hours following a 4 second exposure to heat. Error bars are standard deviations.

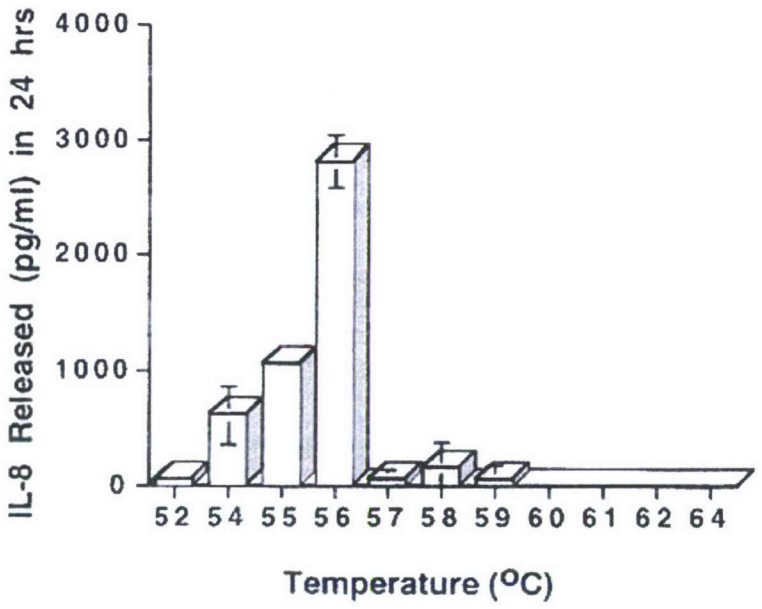


Fig. 6

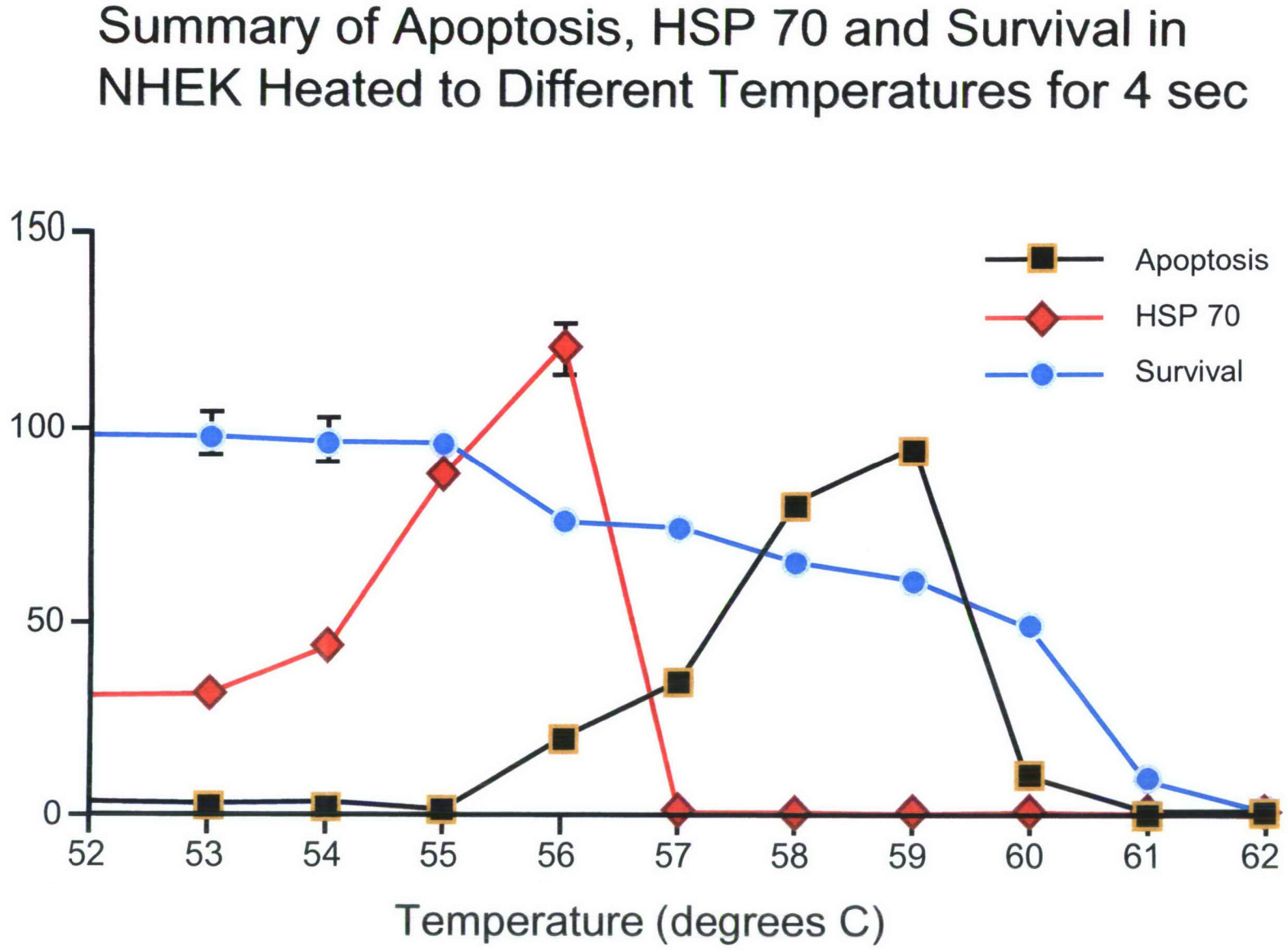

Summary of Apoptosis, HSP 70 and Survival in NHEK Heated to Different Temperatures for 4 sec
150
100
50
0
52
53
54
55
56
57
58
59
60
61
62
Temperature (degrees C)
Apoptosis
HSP 70
Survival

Figure 7

Simultaneous LIVE/DEAD™ and TUNEL Assays for discriminating between apoptosis and ACD.(Right and left micrographs are the same cells.) Photomicrographs of control NHEK (A,B) and NHEK exposed to 58°C (C,D), 60°C (E,F), 62°C (G,H) and 72°C (I,J) for 1 second, 24 hours posttreatment. Staining was withLIVE/DEAD™ viability/cytotoxicity kit (A,C,E,G,I) to differentiate live (green) from dead (red) cells followed by TUNEL labeling (B,D,F,H,J) to indicate cells with fragmented DNA (red). Small arrows indicate viable cells with fragmented DNA. Large arrows indicate dead TUNEL positive cells.

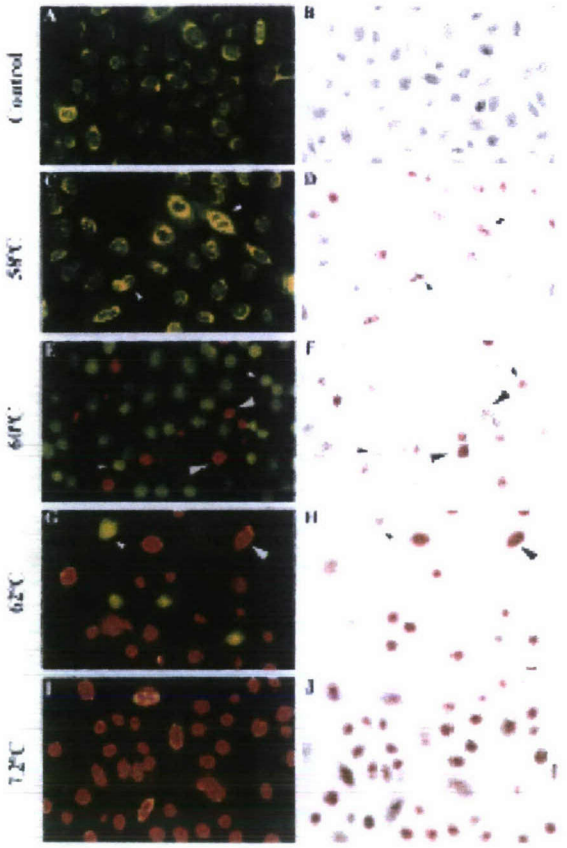


Figure 8

- MitoTracker™ Orange evaluation of mitochondrial function after thermal injury 24 hours posttreatment. Photomicrographs of control NHEK (A,D,G,J) and cells heated to 58°C (B,E,H,K) or 62°C (C,F,I,L). Stainning with calcein AM (A,B,C) to identify live (green) cells and MitoTracker™ Orange (D,E,F) to visualize functional mitochondria (bright orange), followed by TUNEL labeling (G,H,I) to mark cells with fragmented DNA (red). J,K,L - Staining pattern of a single cell with MitoTracker™ Orange.

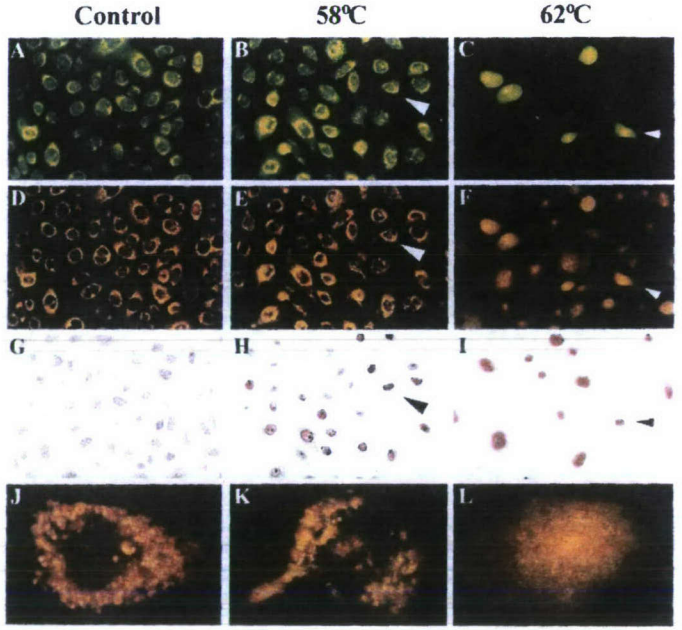


Figure 9

Transmission electron micrographs of thermally injured (58°C for 1 second) NHEK, 24 hours posttreatment. A) Final stages of apoptosis by ethidium homodimer staining demonstrating a viable but fragmenting cell. B) A apoptotic cell by TEM. C. TEM of an apoptotic cell being phagocytosed by a less injured one.

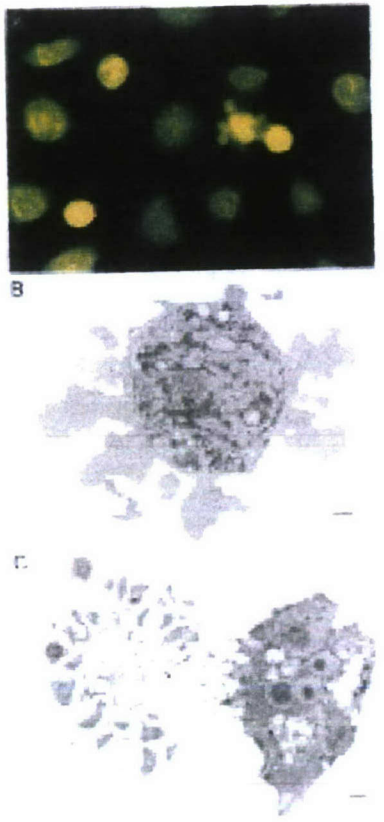


Figure 10

Transmission electron micrographs of mitochondria in control or thermally injured NHEK. Control (A); twenty hours after exposure to 58°C for 1 second (B); twelve hours after exposure to 60°C; (C); or 24 hours after a 1 second exposure to 60°C (D).

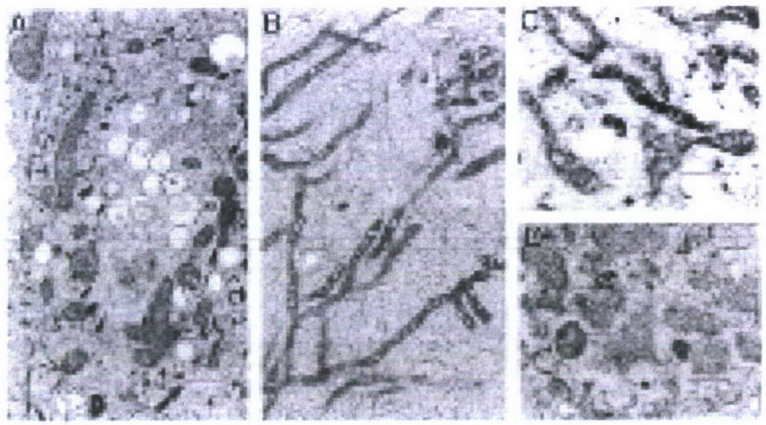


Figure 11

Survival of pretreated versus control NHEK after exposure to heat. Pretreatment consisted of exposure to 55°C for 4 seconds 6 hours before heating.

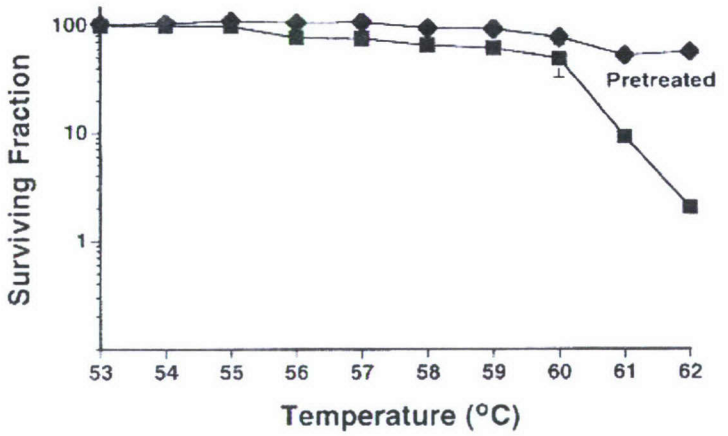


Figure 12

- ARPE-19 cell viability by the Alamar Blue fluorescence assay 24 h after heating at 55°C for 0 to 9 s ($n = 3$). Cell viability is expressed in terms of percent of heat-treated cells surviving compared with unheated control cells.

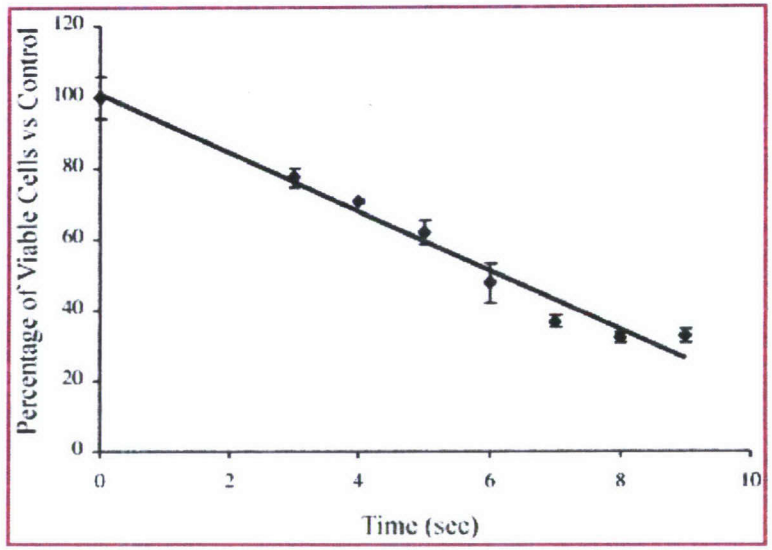


Figure 13

Gene clustering dendrograms and average fold change graphs. Genes undergoing 2-fold change in regulation are cluster analyzed. Each dendrogram contains genes, which have similar functionality and are grouped by temporal expression. Clustering within each dendrogram shows which genes are regulated in a similar manner over the 24-h time course

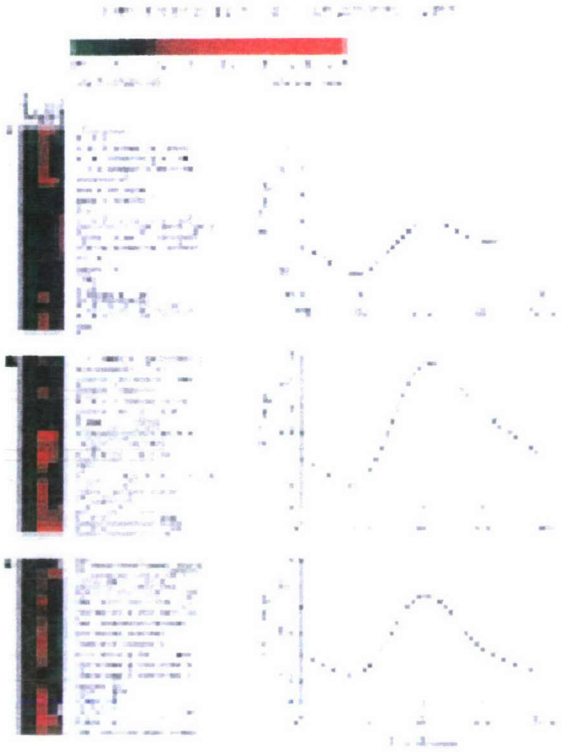


Figure 14

ARPE-19 cell viability 24 h following thermal insult at 55°C for 6-8 s (n=15) with and without pretreatment with HA. Cell viability was assessed by the Alamar BlueÔ fluorescence assay and expressed as a percent of heat-treated cells surviving compared with unheated control cells and cells pretreated with 0.10-1.00 g/ml HA (*P<0.05, **P<0.01 for one-way ANOVA compared to 0 g/ml HA untreated/unheated cells, per time point).

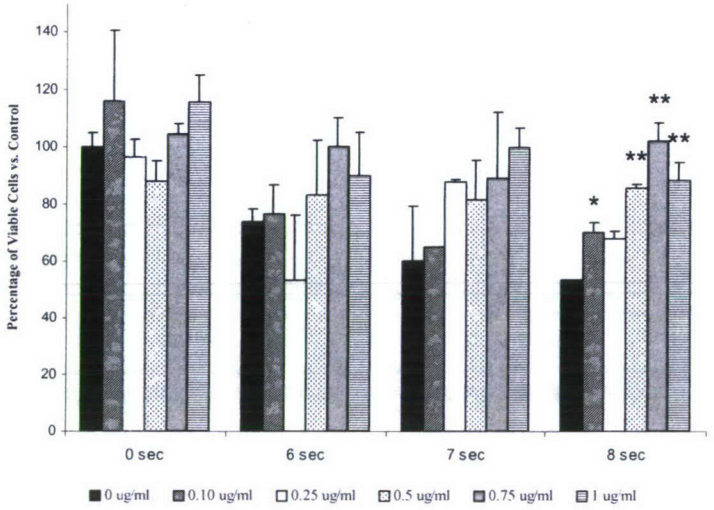


Figure 15

Gene clustering dendogram for ARPE-19 cells treated with 1.00 µg/ml HA. Genes undergoing ≥ 3-fold change in regulation for at least one time point were cluster analyzed.

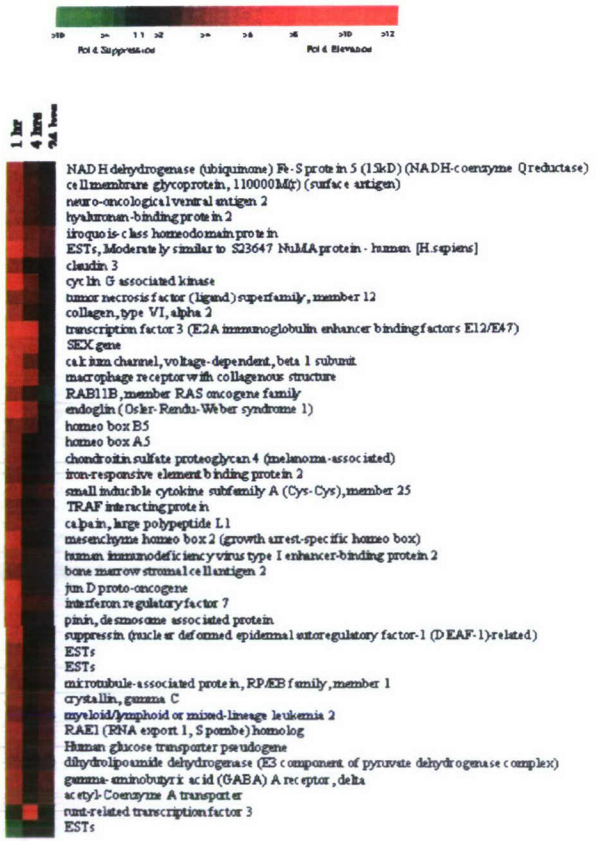
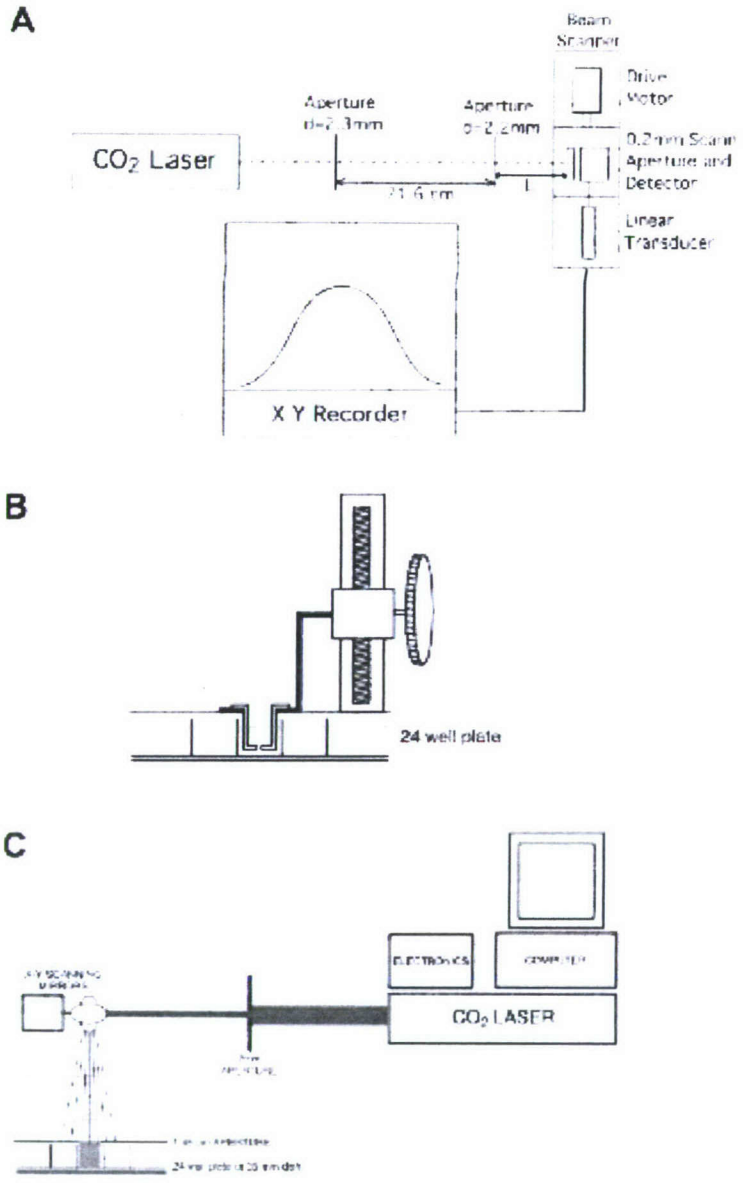


Figure 16



A: schematic of laser setup for determination of beam profile. *B*: schematic of configuration of apparatus used to determine the tophat profile of the laser beam at successive distances from a 2.2-mm aperture. *C*: schematic showing setup for laser raster scanning of large numbers of cultured cells in monolayers.

Figure 17

Protection from CO_2 laser injury with herbimycin A (1.0 ug/ml). Cells at 95% confluence.